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ELISA-LIKE FORMAT FOR COMPARING DNA CAPTURE ELEMENTS (APTAMERS) TO ANTIBODY IN DIAGNOSTIC EFFICACY

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DNA Capture Elements (DCEs), or aptamers, are small pieces of artificial DNA (30-60 base pairs) that are selected by increasing stringency for binding to ligand targets varying from pure proteins, lipids, carbohydrates to whole microbes. Due to this stringency of selection, they are expected to have binding constants in the nanomolar range. However, the real test of their efficacy is based on performance in a standard immunoassay-like format. For this purpose, the microtiter, heterologous phase, ELISA-like sandwich assay test was chosen. The antigen of choice was the standard antigen used in commercially available agglutination tests for *Francisella tularensis* (tularemia bacterium). In addition, data were collected using the antigen available from the DoD Biodefense Critical Reagents Program and whole vaccine strain *Francisella tularensis* from the Centers for Disease Control and Prevention (CDC). Microtiter plates were coated with 500 pmol of "polyclonal" capture DCEs (25 total distinct DCE sequences). This minimal amount was able to detect as little as 250 ng of the antigen. The binding was detected using DCE/horseradish peroxidase conjugate indicator and a colorimetric and spectrophotometric ABTS test.

INTRODUCTION

DCEs are made by a modification of the method for making specific antiligands (aptamers) from nucleic acids by the systematic evolution of ligands by exponential enrichment (SELEX) method. [1-4] The intent is for DCEs to replace antibodies because of the obvious advantage of not requiring animals for production or proteins with less stability and because of scalability based on mass production of the DCEs in vectors (plasmids) in bacterial hosts that would meet military demands for Biological Defense in a timely fashion.[5,6]

They can be synthesized chemically, forming a large set of potential binding molecules and then selected by stringent affinity conditions. Once selected from the large set by rounds of affinity and polymerase chain reaction amplification, they can be cloned into plasmids of bacteria such as *E. coli* for mass production.[5,6] We also demonstrate their specificity and sensitivity in comparison to commercially available antibody.[7]

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MATERIALS AND METHODS

The methods for making DCEs have been described previously in patents or patent applications now pending.[5-7]

Materials. *Francisella tularensis* killed bacteria and its antiserum were purchased from BD, Biosciences, the second batch of *F. tularensis*, a vaccine strain was obtained from CDC (live vaccine strain Lot # 11, NDBR 101 Lot 0200) and the third batch was received from the Critical Reagent Collection of the Department of Defense Joint Program in Chemical and Biological Defense. Bovine serum albumin, ovalbumin, lysozyme and other chemicals were from Sigma. Synthetic random DNA library, biotin labeled primers and other primers were from Sigma/Genosys. Reagents for PCR were purchased from PGC (Frederick, MD) and Applied Biosystems (Faster City, CA).

Random library and primers. A 102 base single-stranded DNA (ssDNA) template containing 42 bases of random sequence flanked by defined primer-binding sites 5'ACC CCT GCA GGA TCC TTT GCT GGT ACC NNNN (N=42) AGT ATC GCT AAT CAG TCT AGA GGG CCC CAG AAT 3' were synthesized by SIGMA/Genosys. The pool was then amplified via polymerase chain reaction and further purified by urea gel electrophoresis. After UV shadowing, ssDNA bands were cut and eluted from the gel. Following precipitation the pool was used for selection.

In Vitro Selection. Iterative rounds of selection and amplification were performed as described previously by Vivekananda and Kiel (1) In brief, to exclude filter binding ssDNA sequences from the pool, the DNA was passed through a 0.45 µm HAWP filter (Millipore, Bedford, MA) and washed with an equal volume of binding buffer containing 20 mM Tris-HCL, pH 7.5, 45 mM sodium chloride, 3 mM magnesium chloride, 1 mM EDTA, 1 mM diothiothreitol (DTT). In the present study, ssDNA pools (500 pmol for initial rounds and 200 pmol for later rounds) were used in the selection process. Single stranded DNA pools were denatured by being heated to 94 ^{0}C, for 3 minutes and then cooled immediately to 4 ^{0}C in binding buffer Selection was performed by incubating ssDNA pools with 100 µg of total bacterial protein (killed bacteria) at room temperature for one hour in binding buffer by gentle rotation. After 1 hour the aptamer-bacterial complex was vacuum-filtered over a HAWP filter at 5 p.s.i. and washed three times with binding buffer. ssDNA retained on the filter was eluted twice with 200 µl of 7 M urea, 100 mM MES [4-morpholine-ethansulfonic acid (pH 5.5) and 3 mM EDTA for 5 minutes at 100 ^{0}C. Eluted ssDNA was then precipitated with an equal volume of isopropyl alcohol. Selected ssDNAs were amplified by PCR and used for next round of selection. After round 10, the pool was cloned using Topo TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced. Finally 25 unique sequences were obtained and used for further analyses.

D*NA Capture Element (****A****ptamer)* ***E****nzyme-****L****inked* ***I****mmobilized* ***S****orbent* ***A****ssay* ***(D(A)ELISA).*** For these sandwich assays 96 well microtiter plates were used. The selected aptamer cocktail was custom coated with a concentration of 500 pmol / well (Pierce Biotechnology, Rockford, IL) and the non-specific sites were blocked with super block from Pierce. Prior to binding assay, the wells were further blocked with 1% BSA in

PBS for 30 minutes at room temperature. Varying concentrations of total bacterial protein of tularemia ranging from 250, 500 ng, 1 and 2 µg were incubated for one hour at room temperature by gentle shaking in 100 ul of binding buffer. After the indicated time, unbound target was removed and washed twice with 0.1 % Tween-20 in PBS (PBS-T). After washing, biotin-labeled aptamer cocktail was then added to the individual wells at 300 pmol concentrations. The plates were incubated at room temperature for another additional hour. Unbound biotin aptamers were removed and washed twice with PBS-T. One hundred µl of 1:1000 dilution streptavidin conjugated horse radish peroxidase (HRP) was added to the individual wells. Following 30 minute incubation at room temperature on a shaking platform, wells were washed twice with PBS-T and developed using ABTS solution (Sigma) according to the manufacture's instructions and read the absorbance at 405 nm. Albumin from chicken egg and lysozyme were used instead of tularemia as negative controls.

ELISA. To compare aptamer cocktail versus anti-tularemia antibodies, we developed a sandwich ELISA assay using commercially available antibodies. Microtiter plate wells were custom coated (Pierce Biotechnology) with anti-tularemia antibodies (BD Biosciences) in desired concentration. All the non-specific sites were blocked by super block. Following further blocking with 1% BSA in PBS, tularemia total protein was added to the wells in the concentrations ranging from 250, 500 pg to 1 and 2 µg in PBS-T. After an incubation of one hour at room temperature, wells were washed with PBS-T for three times. After removal of unbound antigen, the wells were incubated with anti-tularemia rabbit IgG at a dilution of 1:1000 for one hour. Following incubation, wells were washed with PBS-T for three times and finally anti-rabbit IgG conjugated to HRP enzyme was added to the wells for 30 minutes. Excess enzyme was removed from the wells and washed thrice with PBS-T and developed with ABTS substrate according to the manufacturer's recommendation. Like in the D(A)ELISA, albumin and lysozyme were used as negative controls.

Western DOT Blot. Two sets of tularemia total protein samples with concentrations ranging from 25 to 50ug were spotted onto nitrocellulose membrane discs and allowed to air dry. These samples were fixed under vacuum at 80 ^{0}C for one hour and were blocked with 5% BSA in PBS. One batch of samples was incubated with biotin-labeled aptamer cocktail in binding buffer for one hour at room temperature by gentle rocking. The other batch was probed with anti-tularemia polyclonal antibody in PBS-T (1:1000) for 1 hour. After the designated time both batches of membrane discs were washed with PBS-T three times and incubated with streptavidin conjugated alkaline phosphatase and anti-rabbit IgG conjugated alkaline phosphatase respectively for 30 minutes. Excess enzyme was removed by three subsequent washes with PBS-T. Finally the membranes were developed with BCIP/NTB-BLUE (SIGMA).

RESULTS

Figures 1 and 2 show the results, with standard errors of the means, using the ELISA and D(A)ELISA microtiter plate assays. Clearly, the DCEs and polyclonal antibody perform at least equally well in these standard assays down to the 250 ng of antigen measured for both commercially available antigen (BD) and Critical Reagent Program antigen (SA). The antibody, as might be expected, shows a better response with the commercially available antigen, to which it was made, than to the Critical Reagent antigen. Surprisingly, the DCEs work equally well with both antigens. In addition, the DCEs show less non-specific binding than the antibody. Figure 3 shows that the aptamer also works well in a Western dot blot assay with whole tularemia bacteria.

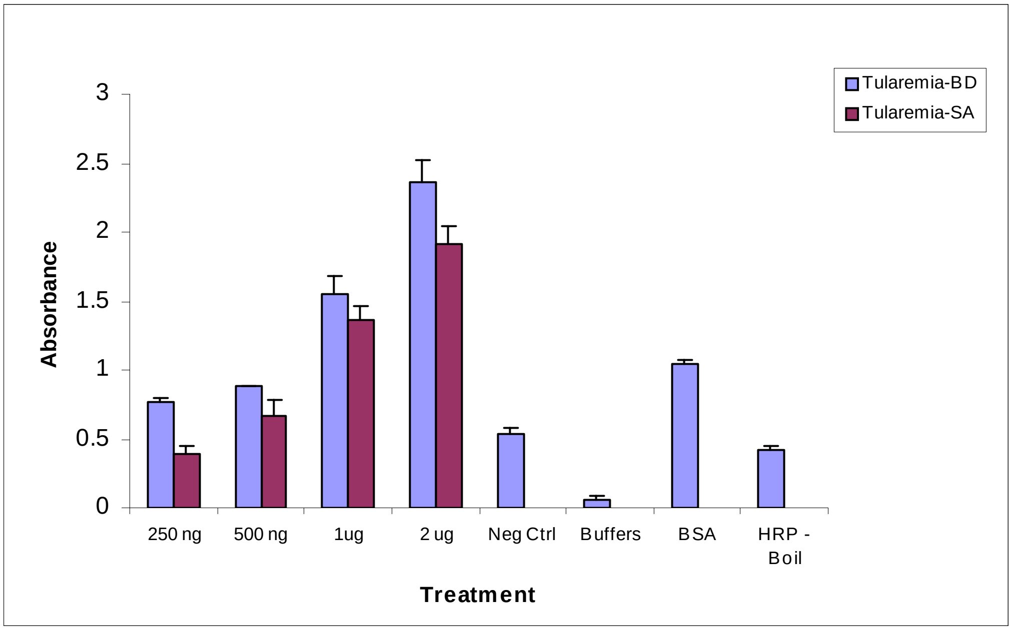

Figure 1. Anti-*Francisella tularensis* polyclonal antibody – ELISA. Neg Ctrl = negative control; HRP-Boil = boiled, inactivated horseradish peroxidase reporter.

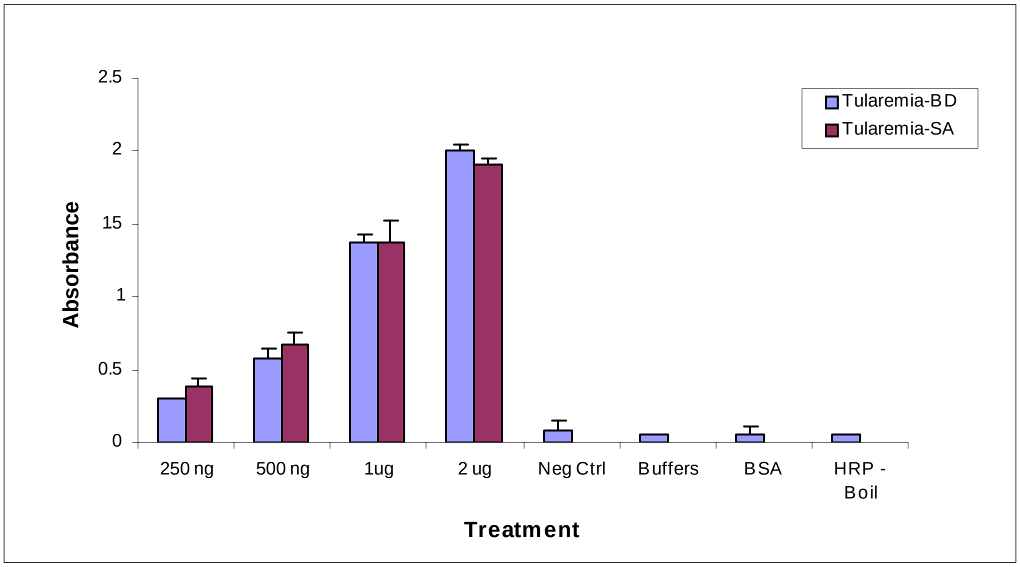

Figure 2. Anti-*Francisella tularensis* aptamer DELISA.

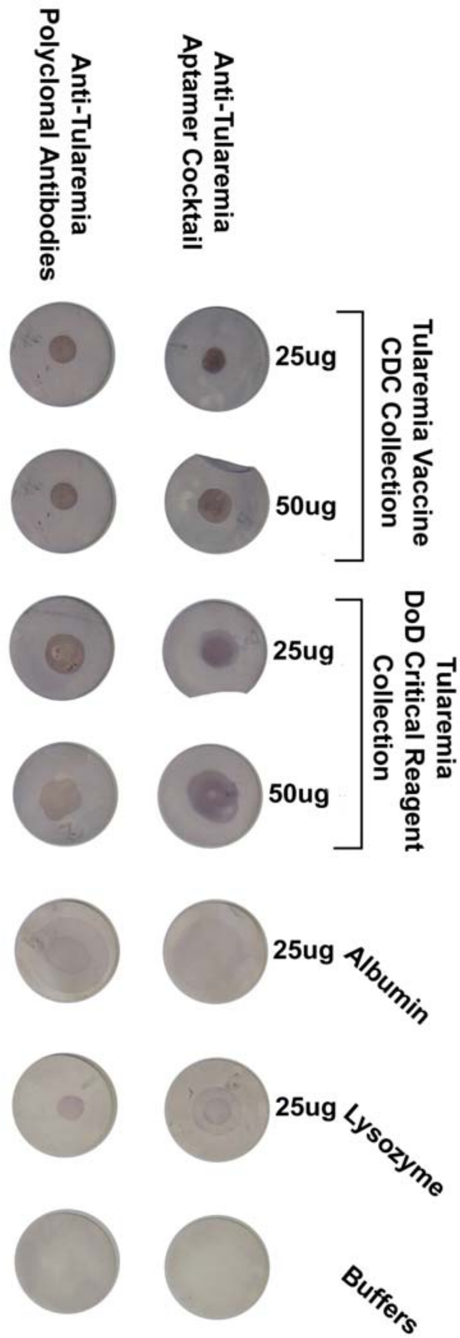

Figure 3. Dot Blot results with whole killed *Francisella tularensis* (vaccine strain) and Critical Reagent Program antigen.

DISCUSSION

We have made steady progress, since the inception of this Joint Service Tech Base Program in Chemical and Biological Defense sponsored project in 1998, toward determining the feasibility of generating DNA capture element-based biological point detectors and identifiers to replace antibody-based devices.[7] We have demonstrated that DCEs perform in ELISA-like assays. They have been directly compared to commercially available antibody in a classic ELISA format assay. This work has also covered a range of agents—toxins, spores, vegetative bacteria, and viruses—indicating its versatility in application.[7] The ongoing work, presented here, with tularemia bacteria further supports this assertion.

CONCLUSIONS

DNA capture elements (DCEs) to four agents (anthrax spores, Shiga toxin, VEE, and tularemia bacteria) have been developed.[7] The DCEs for anthrax spores, tularemia bacteria, and Shiga toxin have been cloned and sequenced. The specificity and sensitivity of the anti-anthrax and anti-tularemia DCEs have been demonstrated and shown to exceed antibody. We have developed an ELISA-like assay for anthrax, and one for tularemia that we directly compared here to one based on antibody. We have shown DCEs neutralize Shiga toxin in vitro, using a human kidney cell target, in turn, demonstrating the potential therapeutic value of DCEs. Finally, we have shown that the neutralization activity is comparable to antibody.

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